



## **Expression of hsa-miR-708-3p and hsa-miR-708-5p in Neuroblastoma cell lines**

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## Abstract

Neuroblastoma (NB) is an aggressive pediatric cancer originating from neural crest cells and is often associated with poor prognosis due to genetic alterations such as *MYCN* amplification and 11q deletion. MicroRNAs (miRNAs) are short RNA molecules that influence gene regulation and play significant roles in tumor formation and progression. This analysis considers the expression of hsa-miR-708-5p and hsa-miR-708-3p in five neuroblastoma cell lines (SH-SY-5Y, NB69, IMR32, SK-N-AS, and SK-N-BE), with and without the above mentioned genetic alterations. Quantitative PCR (qPCR) was used to measure miRNA expression, with U6B small nuclear RNA (RNU6B) serving as an endogenous control. In this study, miR-708-5p was detected in all neuroblastoma cell lines, while miR-708-3p expression was observed only in NB69, SH-SY-5Y, and IMR32. The 11q deletion in SK-N-AS may be the possible reason for the absence of miR-708-3p, although miR-708-5p was still expressed, likely due to a heterozygous deletion that left intact copies of the gene. In SK-N-BE, the lack of miR-708-3p expression may result from epigenetic silencing, chromosomal alterations affecting the miRNA locus, or natural absence of expression under the studied conditions. Statistical analysis confirmed significant expression differences in both miRNAs. ANOVA showed lower miR-708-5p expression in the 11q-deleted group, while Welch's t-test revealed reduced miR-708-3p expression in IMR-32 versus control, indicating biologically relevant variation. Expanding future studies with larger sample sizes across different cell lines is necessary to better understand the complex regulation of miR-708-5p and miR-708-3p in neuroblastoma and to explore their potential as biomarkers or therapeutic targets.

## Popular scientific summary

Neuroblastoma is a rare and aggressive cancer that primarily affects young children, with most cases occurring in those under 10 years old. This cancer develops from neural crest cells, which are responsible for forming the peripheral nervous system. Neuroblastoma accounts for about 8% of all childhood cancers and is known for its wide range of clinical and biological behaviors. While some forms of neuroblastoma are treatable, others are more aggressive and harder to manage, especially those linked to specific genetic changes. Among these, *MYCN* amplification and 11q deletion are two key genetic markers associated with high-risk neuroblastoma and poorer patient outcomes. These genetic alterations contribute to aggressive tumor growth, resistance to therapies, and worse survival rates.

This study investigates the role of microRNAs (miRNAs) in neuroblastoma. miRNAs are small, non-coding RNA molecules that regulate gene expression by binding to messenger RNAs (mRNAs), preventing their translation into proteins, or promoting their degradation. miRNAs are involved in essential cellular processes, such as growth, survival, and repair, and disruptions in their function can contribute to cancer. Tumor-suppressing miRNAs can block the expression of oncogenes and other cancer-promoting pathways, making them potential targets for cancer therapy. The study focused on examining the expression of miR-708-5p and miR-708-3p in five neuroblastoma cell lines (SH-SY-5Y, IMR32, SK-N-AS, SK-N-BE, and NB69) to see how their levels might vary based on genetic features such as *MYCN* amplification and 11q deletion. The expression levels of these miRNAs were measured using quantitative polymerase chain reaction (qPCR), a common technique for accurately quantifying RNA. The results showed that both miR-708-5p and miR-708-3p had higher expression levels in the IMR32 cell line. In contrast, miR-708-5p had undetectable expression in SK-N-AS, while miR-708-3p was not detected in SK-N-AS and SK-N-BE cell lines. Statistical analysis confirmed significant expression differences in both miRNAs. ANOVA showed lower miR-708-5p expression in the 11q-deleted group, while Welch's t-test revealed reduced miR-708-3p expression in IMR-32 versus control, indicating biologically relevant variation, but the results should be interpreted with caution due to the small sample size.

Further research with more cell lines and improved methodologies is needed to better understand the role of these miRNAs in neuroblastoma and to assess whether targeting them could be a viable therapeutic strategy. miRNAs often function within complex regulatory networks, and their activity can vary depending on the context. To fully understand the impact of miRNA deregulation in neuroblastoma, additional functional studies are necessary to explore how these molecules influence tumor growth, survival, and response to treatment.

These findings contribute to the growing body of knowledge about the molecular mechanisms underlying neuroblastoma and emphasize the importance of miRNAs in cancer research. Future studies could investigate the potential of miR-708-5p and miR-708-3p as diagnostic markers or therapeutic targets. In particular, research aimed at uncovering their target genes and pathways could provide valuable insights into their roles in neuroblastoma progression and treatment response. Ultimately, these efforts could lead to the development of new treatment strategies that improve outcomes for children with this aggressive disease.

## Abbreviations

11q	Chromosome 11 Long Arm
ATCC	American Type Culture Collection
CCM	Cell Culture Medium
cDNA	Complementary DNA
CT	Cycle Threshold
FBS	Fetal Bovine Serum
FC	Fold Change
HEPES	N-2-hydroxyethyl piperazine- N'-2-ethane sulfonic acid
hsa-miR-708-5p	Homo sapiens MicroRNA 708-5p
hsa-miR-708-3p	Homo sapiens MicroRNA 708-3p
INRG	International Neuroblastoma Risk Group
miRNA	MicroRNA
<i>MYCN</i>	<i>MYC</i> proto-oncogene, N-Myc
NB	Neuroblastoma
NTC	No Template Control
PBS	Phosphate Buffered Saline
PEST	Penicillin/Streptomycin Solution
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNU6B	U6B small nuclear RNA
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
snRNA	Small nuclear RNA

## Contents

Introduction .....	1
Neuroblastoma .....	1
<i>MYCN</i> amplification and 11 q deletion .....	2
MicroRNAs .....	3
Aim .....	5
Objectives .....	5
Method .....	6
Cell culture .....	6
miRNA isolation .....	7
Reverse Transcription .....	7
Quantitative Polymerase Chain Reaction (qPCR) amplification .....	7
qPCR data treatment prior to analysis .....	8
qPCR data analysis .....	8
Results .....	9
Cell culture .....	9
Total RNA isolation .....	9
RT and qPCR .....	11
qPCR data Analysis .....	12
Discussion .....	14
Cell culturing .....	14
Small RNA Isolation .....	14
Analysis of miR-708-5p and miR-708-3p expression levels .....	15
Conclusion .....	18
Ethical considerations .....	19
Acknowledgements .....	22
References .....	23
Appendix I .....	30

## **Introduction**

### **Neuroblastoma**

Neuroblastoma (NB), a tumor originating from the sympathetic nervous system, stands as the predominant extracranial solid tumor in childhood, usually under the age of five. Accounting for approximately 7–8% of all childhood malignancies and contributing to 15% of all cancer-related deaths in this population, neuroblastoma holds a prominent position in pediatric oncology (Weinstein et al., 2003). It is believed to develop from precursor cells that are in the process of maturation and originate from neural-crest tissues. Common locations include the adrenal medulla or paraspinal ganglia, leading to mass formations in the neck, chest, abdomen, or pelvis (Maris, 2010). The most commonly observed metastatic sites include regional lymph nodes, bone marrow, bone, liver, and subcutaneous tissue (Weinstein et al., 2003; Mlakar et al., 2017). Most primary tumors occur within the abdomen, with at least 49% arising from the adrenal glands (Lanza et al., 2019)

The clinical presentation varies widely, ranging from asymptomatic masses to primary tumors causing significant health issues due to local invasion, widespread dissemination, or a combination of both factors. The general symptoms in NB are malaise, fever, and weight loss. Others might present with an enlarging mass, pain, abdominal distension, swollen lymph nodes, or respiratory distress due to compression or an enlarged liver (Park et al., 2008; Brodeur & Bagatell, R., 2014). Pelvic masses can lead to constipation or difficulty urinating, whereas involvement in the thoracic region might cause difficulty swallowing, shortness of breath, or, in rare cases, thoracic outlet syndrome (Colon & Chung, 2011).

The incidence of neuroblastoma is 10.2 cases per million children under 15 years of age, making it the most frequently diagnosed cancer during childhood (Maris, 2010), and around 600 cases are annually reported from the US population. Although 90% of cases are diagnosed before the age of 5, 30% of these are identified within the first year. The median age at diagnosis is 22 months (Esiashvili et al., 2009). It is uncommon for the condition to manifest during adolescence and adulthood, but the prognosis is significantly worse in these older age groups (Colon & Chung, 2013).

NB is stratified by the International Neuroblastoma Risk Group (INRG) into distinct categories comprising low, intermediate, and high risks based on specific clinical and biological factors (Cohn et al., 2009). While individuals diagnosed with low- and intermediate-risk NB generally exhibit favorable prognoses, those with high-risk NB pose significant treatment challenges, leading to notably elevated mortality rates (Meany 2019), as asymptomatic NB patients categorized as low-risk demonstrate survival rates above 85%, with observation or surgery alone typically recommended as treatment. For intermediate-risk patients, survival rates range between 50 and 75%. These patients typically undergo moderate doses of chemotherapy, with adjustments based on treatment response, along with surgery to remove tumor tissues (Cohn et al., 2009). Patients classified as high-risk have survival rates under 50% and are generally managed with a combination of chemotherapy, surgery, radiation, stem cell transplantation, and immunotherapy (Tolbert & Matthay, 2018).

Tumors with near-diploid or near-tetraploid karyotypes are linked to a poorer prognosis. These tumors are generally more aggressive, with an increased likelihood of rapid growth, metastasis,

and resistance to treatment. Two predominant high-risk subtypes of neuroblastoma are characterized by *MYCN* proto-oncogene amplification and unbalanced 11q-deletion with loss of heterozygosity (LOH), both of which present significant challenges to current treatment strategies (Brodeur, 2003; Carén et al., 2010; Jurcevic et al., 2022). Key genes located on chromosome 11q that may contribute to this increased risk include TP53, a crucial tumor suppressor involved in cell cycle regulation; CDKN1B (p27), which inhibits cell cycle progression; and NTRK3, which plays a role in neuronal differentiation (Cohn et al., 2009; Maris et al., 2007). The loss of these genes can disrupt normal cellular functions, leading to enhanced tumorigenicity and poor clinical outcomes (Cheung et al., 2012). *MYCN* amplification is observed in approximately 20% of neuroblastoma cases. This genetic alteration is correlated with an aggressive disease phenotype, resistance to treatment, and a grim prognosis (Epp et al., 2023). NBs with 11q chromosomal deletions, typically ranging from 11q14.1 to 11qter, are associated with a poor prognosis (Cohn et al., 2009; Mlakar et al., 2017).

Approximately 20% to 25% of neuroblastoma cases exhibit these deletions, which correlate with advanced disease stages and increased treatment resistance, leading to unfavorable outcomes (Cohn et al., 2009).

### ***MYCN* amplification and 11 q deletion**

The *MYCN* proto-oncogene is a member of the *MYC* family of transcription factors, with a critical role in cellular regulation (Liu et al., 2021). *MYCN* amplification is a hallmark of high-risk neuroblastoma and is associated with aggressive tumor behavior and poor prognosis (Brodeur et al., 1984; Kohl et al., 1983). The gene drives cell proliferation by activating the transcription of genes essential for DNA synthesis and cell cycle progression while inhibiting apoptosis through modulation of both pro- and anti-apoptotic proteins (Cohn et al., 2009; Wang et al., 2014). This dual function allows cancer cells to evade programmed cell death, a key hallmark of oncogenesis (Hanahan & Weinberg, 2011). Additionally, *MYCN* contributes to metabolic reprogramming by upregulating glycolytic pathways, thereby meeting the high energy demands of rapidly dividing cells (Bansal et al., 2022). Clinically, *MYCN* amplification serves as a diagnostic and prognostic indicator, most notably identified through fluorescence in situ hybridization (FISH) analysis, which has become a standard tool for evaluating high-risk neuroblastoma cases (Taylor et al., 2000). Its presence at diagnosis strongly correlates with poor clinical outcomes, making *MYCN* amplification a critical focus in understanding neuroblastoma biology and developing targeted therapies.

Chromosome 11q deletion, which entails the loss of genetic material on the long arm of chromosome 11, is a significant genetic alteration in aggressive NB and is strongly associated with advanced disease stages and poor prognosis (Maris et al., 2007). The deletions are often accompanied by unbalanced loss of heterozygosity (LOH), which leads to the inactivation of tumor suppressor genes and genomic instability, further driving tumor progression (Mlakar et al., 2017; Jurcevic et al., 2022). In this chromosomal region harbors several microRNAs, including miR-708-5p and miR-708-3p, which are believed to regulate genes involved in cell cycle control and apoptosis. The loss or reduced expression of these miRNAs due to 11q deletions may further impair tumor suppressor activity, contributing to the aggressive nature of neuroblastoma (Jurcevic et al., 2022).

11q deletion and *MYCN* amplification are rarely coexist, indicating that they define biologically distinct subtypes of NB (Keller et al., 2022).

## **MicroRNAs**

MicroRNAs (miRNAs) constitute a class of small, non-coding RNA molecules, typically 18–24 nucleotides in length, present in plants, animals, and viruses (Bartel, 2004). They serve pivotal functions in diverse biological processes, such as development, cell differentiation, and the maintenance of cellular homeostasis. Acting as post-transcriptional regulators, miRNAs modulate gene expression by binding to target messenger RNAs, leading to their degradation or inhibiting translation (Lewis et al., 2003; Bartel, 2009). This regulatory role underscores the significance of miRNAs in orchestrating intricate cellular mechanisms and highlights their potential as key players in understanding and manipulating fundamental biological processes, as well as in the development of therapeutic interventions for various diseases (Brennecke et al., 2003).

Humans possess around 2,588 miRNAs that play a vital role in the regulation of over 60% of human genes (Shu et al., 2017). Their expression level changes have been associated with various diseases, including cancer, making them significant molecules in both normal cellular function and pathological conditions, so they are used as diagnostic and prognostic markers in cancer. They have different expression patterns between normal and cancerous tissues (Carén et al., 2010). During miRNA processing, a precursor hairpin (pre-miRNA) gives rise to two strands. The 5p strand, derived from the 5' arm, and the 3p strand, derived from the 3' arm (Kim et al., 2009). Although usually only one strand is active, in many cases but especially in cancer, both the 5p and 3p strands can be active and may control different target genes (Krol et al., 2010). In NB, several miRNAs have been shown to influence tumor behavior. For instance, miR-23a enhances NB cell migration by targeting cadherin-1 (Cheng et al., 2014), miR-338-3p suppresses NB cell proliferation (Chen et al., 2013). Additionally, miRNA expression is regulated by oncogenes such as *MYCN*, which is frequently amplified in high-risk NB. *MYCN* can suppress tumor-suppressive miRNAs while promoting oncogenic ones, thereby creating feedback loops that sustain tumor growth and therapy resistance (He et al., 2014).

According to Jurcevic et al. (2022), 26 miRNAs including miR-708-5p and miR-708-3p located within the 11q14-qtel region have been implicated in NB development (Fig 1). Their research employed KEGG pathway analysis to identify biological processes influenced by these miRNAs, uncovering several enriched cancer-related pathways, including the Hippo, PI3K, and FoxO pathways. Their comprehensive bioinformatics analysis showed that these miRNAs target genes involved in 61 KEGG pathways, particularly those related to cell proliferation, evasion of apoptosis, and DNA repair mechanisms (Bown et al., 1999; Jurcevic et al., 2022). This study focus on miR-708-5p and miR-708-3p, which originate from the same precursor located within the intron of the *ODZ4* host gene (Jurcevic et al., 2022).

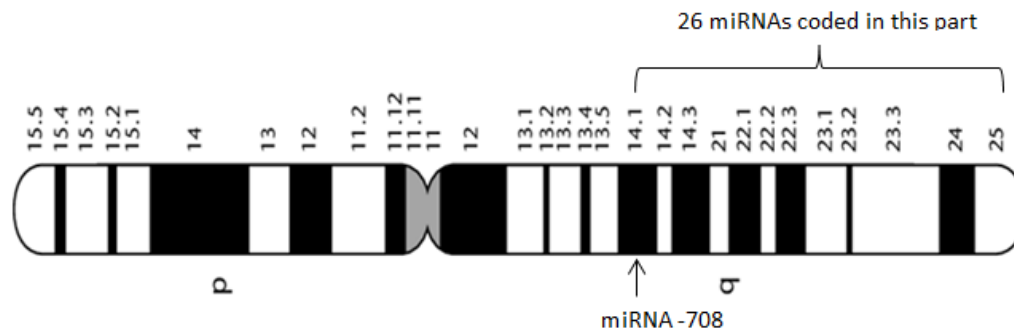


Figure1. The image shows the chromosomal positions of miR-708-5p and 3p within the 11q14.1 region.

miR-708-5p is widely recognized as a tumor suppressor in several cancers, including prostate, breast, and lung cancer. It plays a role in regulating genes involved in apoptosis and cell cycle control, and its expression has been associated with increased sensitivity to chemotherapy and reduced tumor aggressiveness (Bo et al., 2022; He et al., 2014). In neuroblastoma, miR-708-5p is predicted to target key components of the PI3K-Akt and FoxO signaling pathways, which are also involved in supporting *MYCN* stability and tumor progression (Smith et al., 2015)). Notably, miR-708-5p is located on chromosome 11q14.1, a region frequently deleted in high-risk neuroblastoma, and its expression tends to be reduced in NB cell lines with 11q deletions (Jurcevic et al., 2022). The expression pattern of miR-708-5p may offer valuable insights for future research, particularly regarding its potential as a biomarker and regulator of *MYCN*-related signaling.

Conversely, while miR-708-3p has been described as a tumor suppressor in some studies, it has also demonstrated oncogenic activity in several cancer types. In bladder cancer, miR-708-3p promotes tumor cell proliferation and invasion by targeting the *ZEB1* gene, a tumor suppressor involved in the regulation of epithelial-to-mesenchymal transition (EMT), which is crucial for metastasis (Mlakar et al., 2017). In lung adenocarcinoma, it targets *TMEM88*, a negative regulator of the Wnt signaling pathway, thereby enhancing cancer cell survival and growth (Xu et al., 2016). The Wnt signaling pathway plays a pivotal role in neuroblastoma by promoting cell proliferation, preventing differentiation, and supporting stem-like characteristics. Its dysregulation, particularly through its functional interaction with *MYCN*, contributes to tumor aggressiveness and therapy resistance (Szemes et al., 2019). Also miR-708-3p is located on chromosome 11q14.1, a region frequently deleted in high-risk NB (Jurcevic et al., 2022). This deletion can lead to reduced expression of miR-708-3p, potentially contributing to tumor progression. Although the target genes of miR-708-3p in neuroblastoma are not yet fully known, its role in regulating the Wnt pathway in NB is still uncertain because there haven't been enough studies so far. Further research is needed to clarify the implications of miR-708-3p expression in NB and its potential as a therapeutic target. The expression patterns of these two miRNAs form in neuroblastoma underscore their distinct biological roles, serving as a prognostic biomarker for treatment response.

In this study, five NB cell lines were utilized to investigate the roles of specific miRNAs and their association with critical genetic alterations, particularly regarding *MYCN* and 11q deletions. SH-SY-5Y cells and NB69 cells which lack *MYCN* amplification and 11q deletions, serve as a baseline

for comparison (Cohn et al., 2009). Those cells, which do not possess *MYCN* amplification or 11q deletions, provide additional insights into miRNA regulation in NB without the confounding effects of these genetic features (Gao et al., 2017). IMR-32 cells are characterized by *MYCN* amplification, making them a valuable model for studying the impact of elevated *MYCN* expression on tumor biology (Pinto et al., 2015) and SK-N-BE cells also show *MYCN* amplification and can be used to further validate findings related to *MYCN*-driven oncogenesis (Yang et al., 2024). Similarly, SK-N-AS cells, which exhibit 11q deletions, allow for the exploration of how this chromosomal alteration affects miRNA expression and tumor behavior (Jurcevic et al., 2022).

Understanding the expression of miR-708-5p and miR-708-3p in NB in 11q deletion and *MYCN* amplification not only enhances our knowledge of tumor biology but also opens avenues for developing novel therapeutic strategies that could significantly improve patient outcomes in childhood cancers.

## **Aim**

The aim of this project was to study the expression levels of , hsa-miR-708-5p and hsa-miR-708-3p, in neuroblastoma cell lines with and without *MYCN* amplification or 11q deletion.

## **Objectives**

1. Culture the five neuroblastoma cell lines need for isolating total RNA.
2. Isolate total RNA, including miRNA, from the cultured cells.
3. To convert miRNA into complementary DNA (cDNA), carry out reverse transcription (RT) on the isolated total RNA.
4. Perform quantitative polymerase chain reaction (qPCR) to measure the expression levels of miR-708-5p and miR-708-3p in the neuroblastoma cell lines using relative quantification.
5. Use GenEx software to compare the expression levels of miR-708-5p and miR-708-3p across cell lines, with normalization against the endogenous control RNU6B using the  $\Delta Ct$  (delta Ct) method.

## Method

### Cell culture

Human neuroblastoma cell lines, including SH-SY-5Y, IMR-32, SK-N-AS, SK-N-BE, and NB69, were obtained from the ATCC Cell Line Collection (ATCC). These cell lines were chosen for their distinct genetic characteristics, such as *MYCN* amplification and 11q deletion, which are significant in studying neuroblastoma biology and therapeutic responses in Table 1.

Table 1: Characteristics of each neuroblastoma cell line

Neuroblastoma Cell Line	<i>MYCN</i> amplification/ 11q deletion
SH-SY-5Y	without <i>MYCN</i> amplification or 11q deletion
NB69	without <i>MYCN</i> amplification or 11q deletion
IMR-32	With <i>MYCN</i> amplification
SK-N-BE	With <i>MYCN</i> amplification
SK-N-AS	With 11q deletion

All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PEST) solution, 1% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, 1% pyruvate, and 1% L-glutamine (all from Sigma-Aldrich). This medium composition supports optimal cell growth and maintains stable culture conditions by providing essential nutrients and buffering agents. The complete culture medium (CCM) was pre-warmed in a 37°C water bath, before starting the cell culture.

Cryopreserved cells were retrieved from liquid nitrogen and quickly thawed in a 37°C water bath for approximately 2 minutes. The thawed cell suspension was placed into a 15 mL tube with pre-warmed CCM and centrifuged at 1000 RPM for 5 minutes. The supernatant was removed, and the resulting cell pellet was gently resuspended in 1 mL of CCM.

The cell suspension was transferred afterward into a T75 culture flask containing 15 mL of pre-warmed CCM and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. After an incubation period of 12–24 hours, the cells were examined under a microscope to assess adherence and growth. If insufficient growth was observed, the CCM was replaced, and the cells were allowed to continue growing under the same conditions. Upon reaching 80–90% confluence, the cells were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich) and detached using 1x trypsin-EDTA (Sigma-Aldrich). The cells were pelleted by centrifugation at 1000 RPM for 5 minutes and stored at -20°C for miRNA isolation. Accurate cell counting was performed using an improved Neubauer counting chamber, which is essential for ensuring precise cell numbers for RNA isolation. This step helps maintain the consistency of experimental setups and ensures that cell counts are reliable. All cell culture procedures were conducted within a Class II Biological Safety Cabinet, which provides a sterile environment to prevent contamination and protect both the cell cultures and the researcher (Freshney, 2016).

To ensure data reliability and reproducibility, three passages were prepared from each cell line.

Each passage acted as a biological replicate, which is crucial for accounting for inherent variability in cell cultures and minimizing the impact of any anomalies in a single culture instance (Freshney, 2016). Utilizing multiple passages enhances the accuracy of experimental outcomes by capturing natural biological variability and reducing the potential effects of passage-specific alterations (Lundholt et al., 2003).

### **miRNA isolation**

The entire cell pellet was used for the isolation of total RNA, including miRNAs; the mirVana™ miRNA isolation kit without phenol (Thermo Fisher Scientific) was utilized. The isolated RNA was subsequently diluted to a concentration of 2 ng/μL and then stored at -20°C to preserve its integrity until further analysis. The concentration and purity of the isolated RNA were assessed using a DS-11+ spectrophotometer (DeNovix) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific). The spectrophotometer provided a measurement of the RNA concentration and assessed purity based on absorbance ratios such as A260/A280 and A260/A230 (Wilfinger et al., 1997). The Qubit Fluorometer provided a more sensitive quantification of RNA through fluorescence-based detection, allowing for accurate measurement of RNA concentrations, even at low levels. Each passage of the neuroblastoma cell lines was processed according to the established protocol to ensure consistency and reliability in the RNA isolation process. This careful adherence to protocol facilitates accurate downstream applications and reliable data analysis.

### **Reverse Transcription**

For convert RNA into cDNA the TaqMan MicroRNA Assays protocol (Applied Biosystems) was used. For each cDNA synthesis reaction, 10 ng of total RNA was used in a 15 μL reaction volume. U6B small nuclear RNA (RNU6B) (Thermo Fisher Scientific) was included during the reverse transcription (RT) process and subsequently served as a housekeeping gene for normalization in quantitative PCR (qPCR) reactions. The RT was carried out using the T professional Basic Gradient Thermal Cycler (Biometra). After reverse transcription, the synthesized cDNA was stored at -20°C until qPCR analysis was performed.

### **Quantitative Polymerase Chain Reaction (qPCR) amplification**

The TaqMan MicroRNA Assays Protocol (Applied Biosystems) in accordance with the manufacturer's instructions was used to performed qPCR. For each qPCR reaction, 1.33 μL of cDNA, approximately equal to 1ng and derived from the RT reaction, was used in a total reaction volume of 20 μL. To ensure accuracy, three technical replicates were performed for each of the three biological replicates derived from five different cell lines and RNU6B, following the manufacturer's instructions. In addition, nine no-template control (NTC) reactions were included per assay plate: three each for miR-708-5p, miR-708-3p, and the endogenous control RNU6B. NTC was included to evaluate reaction specificity and detect any potential contamination. The AriaMx Real-Time PCR System (Agilent) was employed for the amplification and quantification of miR-708-5p, miR-708-3p and RNU6B.

The TaqMan™ Small RNA Assay (20X stock) (Thermo Fisher Scientific) specific primers of miRNAs and endogenous Control RNU6B were used for the analysis with the probe (the FAM dye-labeled probe) necessary to amplify and detect the small RNA or miRNA targets.

## **qPCR data treatment prior to analysis**

For each qPCR triplicate, the quantification cycle (Ct) values were recorded for the target miRNAs, miR-708-3p and miR-708-5p, and RNU6B. The Ct value represents the cycle number, at which the fluorescence signal exceeds a threshold, indicating the presence of the target nucleic acid (Schmittgen & Livak, 2008). To ensure the accuracy and reliability of the data obtained from these qPCR assays, several quality control criteria were employed.

If any triplicate set showed two or more missing Ct values, or if the difference between the highest and lowest Ct values within the set exceeded 0.5 cycles, that triplicate was deemed unreliable. Consequently, the qPCR experiment for that triplicate was repeated to obtain more consistent and reliable data. This approach adheres to the protocols recommended by de Ronde et al. (2017), which stress the necessity of maintaining consistency and minimizing variability in qPCR measurements.

The average of the remaining two Ct values was used to replace the missing value when a triplicate contained only one missing Ct value. This practice is designed to preserve the overall integrity of the data analysis, reducing the potential impact of incomplete data and ensuring that the results remain as accurate and representative as possible (Nolan et al., 2006). By following these stringent quality control measures, the reliability of the qPCR data is significantly enhanced, allowing for more robust and valid interpretations of miRNA expression levels.

## **qPCR data analysis**

The qPCR data were analyzed using R software 4.3.1 (2023). Data normalization was carried out using the  $\Delta$ Ct (delta Ct) method, with expression levels normalized to the endogenous control RNU6B.

To calculate fold change (FC) in miRNA expression the  $\Delta\Delta$ Ct method (Livak & Schmittgen, 2001) was used. To calculate the relative expression levels of miR-708-5p and miR-708-3p, NB69 and SH-SY-5Y were used as control cell lines. Since NB69 and SH-SY-5Y share similar genetic characteristics, setting them as the reference ensured consistency in comparison. The mean  $\Delta$ Ct values for NB69 and SH-SY-5Y were calculated separately for each passage. Then, the overall control  $\Delta$ Ct was determined by averaging the mean  $\Delta$ Ct values of NB69 and SH-SY-5Y across three passages.

Using this mean control  $\Delta$ Ct as the baseline, the  $\Delta\Delta$ Ct values for other cell lines were determined by comparing their individual  $\Delta$ Ct values to the mean control  $\Delta$ Ct. Finally, FC values were calculated based on the  $\Delta\Delta$ Ct values, providing a measure of relative miRNA expression across the different neuroblastoma cell lines. Given the small sample size ( $n = 3$  per group), statistical power is limited, which must be considered when interpreting the results. With such few data points, testing for normality is statistically unreliable (Ghasemi & Zahediasl, 2012), for miR-708-5p, expression levels were compared across groups using one-way ANOVA assuming the data were normally distributed and miR-708-3p was expressed in only two groups, so independent welch test was used and assumed the normality. A significance threshold of  $p < 0.05$  was considered statistically significant.

## **Results**

### **Cell culture**

Each cell line was meticulously cultured until reaching 80–90% confluence under controlled conditions. To generate biological replicates, each cell line underwent three subculture cycles. After each passage, cells were counted to determine the appropriate volume of lysis/binding solution for the miRNA isolation procedure. Appendix I, Table 1 provides the cell counts at the time of collecting for each cell line across various passages.

### **Total RNA isolation**

From three different passages of five neuroblastoma cell lines, total RNA—including miRNAs—was extracted for analysis : SH-SY-5Y, NB69, IMR32, SK-N-AS, and SK-N-BE. The RNA quantity and purity were evaluated using a Qubit 3.0 Fluorometer (ThermoFisher Scientific) and a DS-11+ spectrophotometer (DeNovix).

The data in Table 2 includes detailed measurements offering a comprehensive overview of the purity and quantification of total RNA, including miRNA, across the different cell lines and passages.

Table 2: RNAs concentrations and purity measurements including miRNA which isolated from five neuroblastoma cell lines

<b>NB cell line and passage no:</b>	<b>RNA concentration (ng/μl)</b>	<b>260/230</b>	<b>260/280</b>
<b>SH-SY-5Y p11</b>	0.8	2.05	0.23
<b>SH-SY-5Y p12</b>	27.2	2.05	1.95
<b>SH-SY-5Y p13</b>	16.7	1.98	1.84
<b>NB69 p11</b>	70.4	1.99	2.02
<b>NB69 p12</b>	108.0	2.02	1.89
<b>NB69 p13</b>	228.0	2.02	1.65
<b>IMR-32 p6</b>	106.0	2.01	1.75
<b>IMR-32 p7</b>	51.2	2.02	2.03
<b>IMR-32 p8</b>	173.2	2.00	1.85
<b>SK-N-AS p15</b>	46.8	2.03	2.02
<b>SK-N-AS p16</b>	116.0	2.03	1.84
<b>SK-N-AS p17</b>	44.0	2.04	1.07
<b>SK-N-BE p6</b>	224.0	2.03	1.28
<b>SK-N-BE p7</b>	70.6	2.08	2.10
<b>SK-N-BE p8</b>	43.8	2.06	1.85

\* Passage numbers are mentioned as p, for example: SH-SY-5Y p11 specifies the SH-SY-5Y cell line at passage 11.

The RNA concentration and purity were assessed using both Qubit and spectrophotometer measurements for each neuroblastoma cell line across different passages. Qubit provided accurate quantification of RNA concentration (ng/μl), while the spectrophotometer measurements purity ratios (260/230 and 260/280) to evaluate potential contamination.

For SH-SY-5Y, NB69, IMR-32, SK-N-AS and SK-N-BE cell lines, RNA concentrations varied across passages, with NB69 p13 was showing the highest Qubit concentration (228 ng/μl) and SH-SY-5Y p11 was the lowest. The 260/230 and 260/280 ratios were used to assess RNA purity. The acceptable range for the 260/230 ratio is typically 2.0–2.2, indicating minimal contamination from salts or solvents, while the 260/280 ratio should ideally fall between 1.8–2.1, mean high RNA quality with minimal protein contamination (Lucena-Aguilar et al., 2016).

Most samples fell within the acceptable purity ranges, confirming good RNA purity. However, some did not meet the expected values, which may affect downstream analyses. The lowest purity was observed in SH-SY-5Y p11, with a 260/280 ratio of 0.23, it is impossible to obtain reliable purity ratios when the nucleic acid concentration is extremely low (Lucena-Aguilar et al., 2016).

## RT and qPCR

Each cDNA synthesis reaction was performed using 10 ng of miRNA. Following reverse transcription, qPCR was conducted to assess the relative expression of miR-708-5p and miR-708-3p, with RNU6B used as the endogenous control. Each sample was analyzed in technical triplicates. For each qPCR reaction, 1.33  $\mu$ L of cDNA was used. Table 3 presents the average Ct values and standard deviations (SD) for miR-708-5p and miR-708-3p and the endogenous control. SD is essential in evaluating the consistency of qPCR Ct values across technical replicates. The raw qPCR Ct values are provided in Appendix II, Table 1.

Table 3: Average Ct values and standard deviation for miRNA miR-708-5p and miR-708-3p and RNU6B across neuroblastoma cell lines.

Cell Name	miR-708-5p (Average Ct)	miR-708- 5p (SD)	miR-708- 3p (Average Ct)	miR-708- 3p (SD)	RNU6B (Average Ct)	RNU6B (SD)
<b>SH-SY-5Y p11</b>	26.23	0.11	36.70	0.01	28.11	0.04
<b>SH-SY-5Y p12</b>	25.73	0.04	34.45	0.10	28.70	0.01
<b>SH-SY-5Y p13</b>	26.25	0.20	36.66	0.15	27.65	0.14
<b>NB69 p11</b>	25.82	0.09	36.84	0.18	30.14	0.10
<b>NB69 p12</b>	23.62	0.05	35.55	0.16	28.35	0.05
<b>NB69 p13</b>	24.43	0.07	35.57	0.07	30.68	0.07
<b>IMR-32 p6</b>	25.67	0.01	36.06	0.15	30.51	0.07
<b>IMR-32 p7</b>	26.30	0.07	34.28	0.12	29.36	0.03
<b>IMR-32 p8</b>	26.39	0.05	No Ct	N/A	32.15	0.14
<b>SK-N-AS P15</b>	30.33	0.29	No Ct	N/A	27.06	0.05
<b>SK-N-AS P16</b>	30.23	0.37	No Ct	N/A	27.87	0.04
<b>SK-N-AS P17</b>	30.23	0.21	No Ct	N/A	26.86	0.01
<b>SK-N-BE p6</b>	26.38	0.20	No Ct	N/A	29.34	0.13
<b>SK-N-BE p7</b>	26.92	0.06	No Ct	N/A	27.83	0.04
<b>SK-N-BE p8</b>	26.95	0.01	No Ct	N/A	29.78	0.17

\*N/A indicates SD values that no Ct value was detectable for hsa-miR-708-3p in those passages.

miR-708-5p showed stable expression across all examined neuroblastoma cell lines, including SH-SY-5Y, NB69, IMR-32, SK-N-AS, and SK-N-BE, with Ct values generally ranging between 23 and 31 (Schmittgen & Livak, 2008; Taylor et al., 2019). In contrast to miR-708-5p, miR-708-3p exhibited low or undetectable expression in neuroblastoma cell lines. It was detected in SH-SY-5Y, NB69, and IMR-32, with high Ct values ranging from 34 to 37, indicating low expression levels and miR-708-3p was not detected (No Ct) in SK-N-AS and SK-N-BE cells. RNU6B was used as the endogenous control for normalization of miRNA expression, with equal amounts of RNA used for all reverse transcription reactions. While its Ct values were generally centered on 30, some variation was observed across biological replicates and cell lines, with the greatest differences seen in IMR-32 and NB69. The NTC included in the qPCR run did not produce any

detectable Ct values that confirming the absence of contamination in the reagents.

### qPCR data Analysis

The results were analyzed using R statistical software (version 4.3.1, 2023). The endogenous control RNU6B was used for calculating the delta Ct ( $\Delta$ Ct) values of both miR-708-5p and miR-708-3p and the normalization compensates for sample-to-sample variations, enabling reliable comparison of target miRNA expression relative to the endogenous control. For  $\Delta\Delta$ Ct analysis, SH-SY-5Y and NB69 (without *MYCN* amplification or 11q deletion) were used as the control group. The average  $\Delta$ Ct of this group was set as the baseline ( $\Delta\Delta$ Ct = 0), and  $\Delta\Delta$ Ct values for other cell lines were calculated relative to this reference. The delta delta Ct ( $\Delta\Delta$ Ct) values were derived relative to the control group for both miRNA-708-5p and miRNA-708-3p in Table 4.

Table 4:  $\Delta$ Ct and  $\Delta\Delta$ Ct values for miR-708-5p and miR-708-3p in cell lines

Cell Line	$\Delta$ Ct (miR-708-5p)	$\Delta\Delta$ Ct (miR-708-5p)	$\Delta$ Ct (miR-708-3p)	$\Delta\Delta$ Ct (miR-708-3p)
SH-SY-5Y	-2.08	0	7.78	0
NB69	-5.10	0	6.26	0
IMR-32	-4.55	-0.96	5.23	-1.78
SK-N-AS	3.00	6.59	**	**
SK-N-BE	-2.23	1.35	**	**

\*\* indicate no Ct detected in the cell line.

Based on the mean  $\Delta\Delta$ Ct values presented in Table 4 for miR-708-5p and miR-708-3p, the relative expression of both miRNAs was shown in Figure 2.

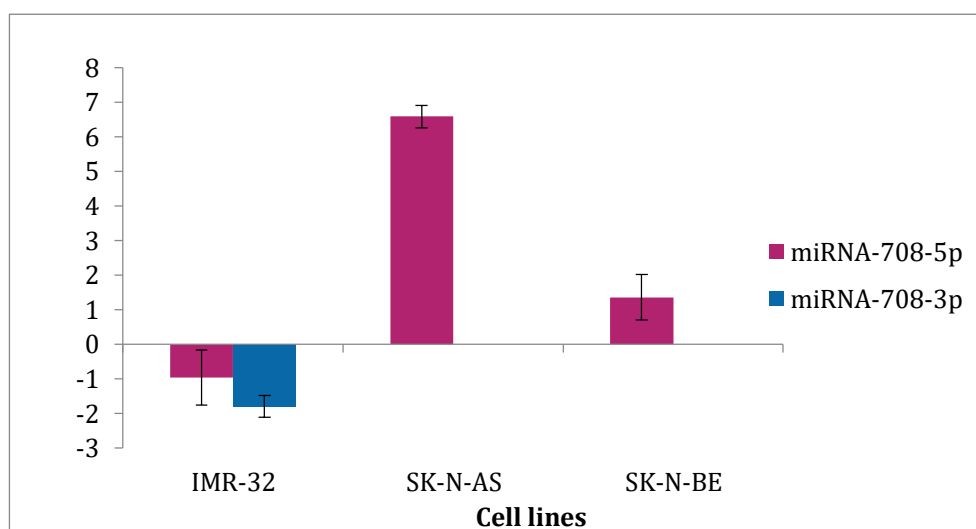


Figure2. Relative expression of miR-708-5p and miR-708-3p across neuroblastoma cell lines using the mean  $\Delta\Delta$ Ct values  $\pm$  SEM. SH-SY-5Y and NB69 were used as  $\Delta\Delta$ Ct = 0, were not included to the plot.

miR-708-5p expression was highest in IMR-32 ( $\Delta\Delta$ Ct =  $-0.96 \pm 0.79$ ), indicating relative higher expression, and lowest in SK-N-AS ( $\Delta\Delta$ Ct =  $6.59 \pm 0.32$ ). For miR-708-3p, expression was detected only in SH-SY-5Y, NB69, and IMR-32, with the highest expression observed in IMR-32 ( $\Delta\Delta$ Ct =  $-1.79 \pm 0.32$ ). No Ct values were detected for miR-708-3p in SK-N-AS and SK-N-BE. A

bar plot with error bars based on  $\Delta\Delta\text{Ct}$  values comparing genetic groups (11q deletion and *MYCN* amplification), using SH-SY-5Y and NB69 as controls, is provided in Appendix I (Figure 1).

To evaluate whether the expression of miR-708-5p significantly differed among neuroblastoma cell lines based on their genetic features, a one-way ANOVA was performed. Cell lines were grouped into *MYCN* amplified (IMR-32 and SK-N-BE), 11q-deleted (SK-N-AS), and non-altered (control) (NB69 and SH-SH-5Y) categories. Table 5 was shown the expression differences of miR-708-5p in *MYCN*-amplified, 11q-deleted, and control neuroblastoma Cell lines.

Table5: Expression differences of miR-708-5p in *MYCN*-amplified, 11q-deleted, and control neuroblastoma cell lines.

Source	Df (Degrees of Freedom)	Sum Sq (Sum of Squares)	Mean Sq (Mean Square)	F Value	p-value
Cell lines	2	94.17	47.08	43.78	<0.0001
Residuals	12	12.90	1.08		

The analysis showed a significant difference between groups ( $p < 0.0001$ ). The high F-value indicates that variation between groups was much greater than the variation within groups. Tukey's HSD post-hoc test was performed to determine which specific groups differed. Table 6 summarized the pairwise comparisons of miR-708-5p expression levels among neuroblastoma cell lines grouped by genetic alterations.

Table 6: Pairwise comparisons of miR-708-5p expression levels among neuroblastoma cell lines grouped by genetic alterations (*MYCN*-Amplified, 11q-Deleted, and Control)

Comparison	Mean Difference ( $\Delta\text{Ct}$ )	95% CI Lower	95% CI Upper	Adjusted p-value
11q vs Control	-6.23	-8.18	-4.27	< 0.0001
11q vs <i>MYCN</i>	-6.30	-8.25	-4.34	< 0.0001
Control vs <i>MYCN</i>	-0.07	-1.67	1.53	0.9925

miR-708-5p expression was significantly difference in the 11q-deleted group compared to both the *MYCN*-amplified and control groups ( $p < 0.0001$ ). There was no significant difference in expression between the *MYCN*-amplified and control groups ( $p = 0.99$ ).

To assess the difference in miR-708-3p expression between the control and *MYCN*-amplified (IMR-32) group, an independent Welch's t-test was performed using  $\Delta\text{Ct}$  values. The result showed  $p = 0.0048$ , there is a statistically significant difference between the control group and *MYCN*-amplified (IMR-32) in miR-708-3p expression.

## Discussion

Aberrant miRNA expression is closely linked to the initiation, progression, and metastasis of cancer. The regulation of miRNA gene expression plays a crucial role in cellular responses to environmental stresses, such as starvation, hypoxia, oxidative stress, and DNA damage, all of which are implicated in cancer development (Otmami & Lewllie, 2021; Chen & Stallings, 2007). In NB, monitoring miRNA levels can provide valuable clinical insights, aiding in tumor differentiation, treatment selection, and outcome prediction (Wang et al., 2018). By certain miRNA profiles can indicate sensitivity or resistance to specific chemotherapeutic agents, thereby guiding personalized treatment plans. Additionally, these expression patterns can offer prognostic value, allowing for better predictions of treatment outcomes (Wang et al., 2018). By closely examining miRNA levels in NB, clinicians and researchers can gain a deeper understanding of the disease's molecular mechanisms, leading to the development of novel therapeutic strategies targeting miRNA pathways. Incorporating miRNA profiling into clinical practice holds promise for improving decision-making and patient outcomes, particularly in aggressive malignancies like neuroblastoma.

## Cell culturing

Five NB cell lines with three distinct genetic characteristics were utilized in this study: SH-SY-5Y (no *MYCN* amplification, no 11q deletion), IMR-32 (*MYCN* amplification), SK-N-AS (11q deletion), SK-N-BE (*MYCN* amplification), and NB69 (no *MYCN* amplification, no 11q deletion). Each cell line was under grown for three passages. Sub culturing is a crucial process in maintaining cell lines, involving the transfer of cells to fresh culture media to support their continued growth and viability. However, repeated passaging can significantly affect cellular characteristics, particularly miRNA expression. miRNAs are essential regulators of gene expression, influencing various cellular processes such as differentiation, proliferation, and stress responses. During sub culturing, factors like enzymatic treatments like trypsinization, mechanical handling, and changes in the culture environment can induce stress, altering miRNA expression profiles (Olejniczak et al., 2017). These changes can lead to variations in cellular behavior and gene regulation across different passages. Therefore, in this study, samples were collected from different passages to monitor and account for possible variations in miRNA expression. Maintaining consistent miRNA expression is essential to ensure the stability of cell lines and the reliability of experimental results. Careful culture management, including minimizing stress and controlling environmental variables, was applied to preserve the integrity of miRNA regulation during subculturing (Lee et al., 2019; Wijk et al., 2022).

## Small RNA Isolation

The concentration and purity of miRNA extracted from various NB cell lines across different passages were evaluated using spectrophotometric methods and Qubit fluorometry, as outlined in Table 2. The ratio of absorbance at 260 nm to 280 nm is a standard measure used to assess the purity of DNA and RNA, with a ratio of around 2.0 generally accepted for pure RNA. Deviations from this ratio may indicate the presence of contaminants such as proteins, phenol, or other substances that absorb strongly at or near 280 nm (Matlock, 2015). Additionally, the absorbance ratio at 260 nm to 230 nm was measured to further evaluate RNA purity, with an accepted range typically between 2.0 and 2.2 (Desjardins & Conklin, 2010; Forero et al.,

2019). The observed variability in miRNA yield and purity suggests that factors beyond cell number, such as extraction efficiency and sample handling, may have contributed to the results (Hu et al., 2020)). Overall, all samples exhibited sufficient concentration and good purity. However, the SH-SY-5Y passage 11 sample showed a very low Qubit concentration (0.78 ng/ $\mu$ L), which likely contributed to the low A260/A280 ratio of 0.23, as spectrophotometric measurements become unreliable at such low RNA concentrations (Imbeaud et al., 2005; Fleige & Pfaffl, 2006). Although a lower value suggesting protein or phenol contamination (Wilfinger et al., 1997), but low RNA concentrations can significantly affect A260/A230 and A260/A280 values, often leading to misleading indications of contamination (Hashemipetroudi et al., 2018). Low concentration of RNA can impact downstream applications like qPCR or RNA sequencing (Sambrook & Russell, 2001). To improve RNA purity, additional purification steps with RNA clean-up kits may be necessary (Chomczynski & Sacchi, 2006). Several strategies can be employed to improve miRNA isolation. Optimizing the lysis buffer composition and ensuring thorough homogenization of cell samples can enhance extraction efficiency. Moreover, using specialized miRNA isolation kits designed to minimize degradation and sample loss can further improve RNA yield and purity (Hu et al., 2020).

### **Analysis of miR-708-5p and miR-708-3p expression levels**

After completing reverse transcription, qPCR was employed to evaluate the relative expression levels of miRNAs 708-5p and 708-3p across various passages of the NB cell lines (Appendix I Table 1).

To ensure reliable qPCR data, three technical replicates were used for each passage. This approach reduces technical variability and enhances the accuracy of Ct measurements, providing a robust assessment of gene expression stability (Livak & Schmittgen, 2001). Replicates help control for potential pipetting errors and instrument variability, ensuring that observed differences reflect biological changes rather than technical inconsistencies (Bustin et al., 2009). The low variation among replicates in this study further supports the reproducibility of results.

The use of RNU6B (U6) as a reference gene in this study warrants critical evaluation. Although U6 is commonly used for normalization in miRNA qPCR assays, its consistently high Ct values (~30) observed across all neuroblastoma cell lines and passages suggest low expression levels and potentially unstable performance. This poses a concern for normalization accuracy, particularly in cancer models where small RNA expression can vary substantially. Peltier and Latham (2008) highlighted that reference gene selection significantly impacts the interpretation of miRNA expression data and emphasized the need to empirically validate reference targets under specific experimental conditions. Furthermore, neuroblastoma cell lines such as IMR-32, SK-N-BE, and others are known to differ markedly in their transcriptional activity and cellular states. Barber et al. (2005) demonstrated that ribosomal and housekeeping gene expression can vary widely across tissues and cell lines, while Sørensen et al. (2010) specifically documented transcriptional differences within neuroblastoma models related to *MYCN* amplification and tumor progression. These findings raise concerns about the stability of U6 in this context. Vandesompele et al. (2002) strongly recommended the use of multiple validated reference genes rather than a single internal control to reduce normalization error and improve the reliability of qPCR data. Taken together, the combined evidence suggests that normalization

using U6 alone may have introduced variability into  $\Delta$ Ct values and miRNA expression measurements. For future experiments, the use of stably expressed endogenous miRNAs validated under experimental conditions or geometric averaging of multiple reference genes, as proposed by Vandesompele et al. (2002), would enhance the robustness and interpretability of results

In the study, both NB69 and SH-SY-5Y cell lines showed detectable Ct values for miR-708-5p and miR-708-3p, confirming the presence of miRNA expression. This result aligns with expectations, as NB69 and SH-SY-5Y lack major genetic alterations such as *MYCN* amplification or 11q deletion.

It was expected to find miR-708-5p and miR-708-3p being expressed in IMR32 and SK-N-BE cell lines which carrying *MYCN* amplification. miR-708-5p is suggested to target components of the PI3K-Akt and FoxO pathways (Bo et al., 2022), which contribute to *MYCN* stability and tumor progression. The regulation of miR-708-3p by *MYCN* has not been fully demonstrated (Jurcevic et al., 2022). In this study, both miR-708-5p and miR-708-3p were detected in IMR32, whereas in SK-N-BE, only miR-708-5p was expressed and miR-708-3p was absent. The lack of detectable miR-708-3p in SK-N-BE may be due to epigenetic silencing, as promoter hypermethylation has been shown to suppress miRNA expression in neuroblastoma (Bray et al., 2009). Although *MYCN* amplification is typically associated with the suppression of tumor-suppressor miRNAs, including miR-708-5p, it was expected that *MYCN* amplified cell lines would exhibit lower expression of this miRNA. Interestingly, in this study, IMR-32 showed higher miR-708-5p expression compared to SK-N-BE, despite both lines carrying *MYCN* amplification. This discrepancy may be due to intrinsic differences in transcriptional regulation or miRNA processing between the cell lines, as neuroblastoma cell lines are known to display significant transcriptional heterogeneity even when sharing oncogenic features (Sorensen et al., 2010). Furthermore, strand-specific miRNA processing may result in differential expression of miR-708-5p and miR-708-3p across cell lines. Technical factors, such as variability in reference gene stability particularly RNU6B, which showed high Ct values could also influence normalization and the interpretation of miRNA expression data (Barber et al., 2005; Vandesompele et al., 2002; Peltier & Latham, 2008).

In the SK-N-AS cell line, which carries an 11q deletion, miR-708-3p was undetectable. The 11q deletion leads to the loss of chromosomal regions encompassing key tumor suppressor genes and miRNA loci, which likely accounts for the absence of these specific miRNAs (Carén et al., 2010; Coronado et al., 2020). These findings underscore the impact of genetic alterations, such as chromosomal deletions, on miRNA expression, contributing to distinct miRNA profiles among different NB cell lines (Machowska et al., 2022). The loss of miR-708-3p in the context of 11q deletion suggests a potential mechanism through which NB cells may escape regulatory control, further complicating the disease's molecular landscape (Maris et al., 2007). Understanding these genetic influences is crucial for elucidating the complex regulatory networks involved in NB and may aid in developing targeted therapies aimed at restoring normal miRNA function (Aravindan et al., 2019).

The miR-708-5p showed detectable expression in SK-N-AS even though it is located within the deleted 11q region (Jurcevic et al., 2022). Its detection could be due to the 11q deletion being partial and leaving some intact miR-708 genes (Maris et al., 2007). In this case, miR-708-5p can

still be transcribed and expressed. Also, genomic instability in SK-N-AS cells could cause different deletion patterns in different cells (Guo et al., 2010). These possibilities suggest that miRNA expression can sometimes still be detected even when chromosomal deletions are present, highlighting the need to check both gene presence and miRNA expression together (Huisman et al., 2022; Winter & Diederichs, 2011). Understanding miRNA expression stability and variation will be valuable for future neuroblastoma studies, especially when considering miRNAs as potential therapeutic targets (Andreeva et al., 2023). Ensuring high-quality of RNA with minimal contaminants is essential for precise reverse transcription and amplification, thereby reducing variability in miRNA expression analysis.

Due to the small number of samples in each group ( $n = 3$ ), formal testing for data normality was not performed, as normality tests like Shapiro-Wilk are unreliable with very small sample sizes (Razali, N. & Wah, Y. 2011). Given the limited sample size but assuming approximate normal distribution, a one-way ANOVA was used to compare miR-708-5p expression levels across groups. One-way ANOVA was selected because it remains relatively robust to mild violations of normality, when group sizes are equal (Blanca et al., 2018). It is revealed a statistically significant difference in the groups; suggesting true biological variation in miR-708-5p expression and post hoc tests indicated that miR-708-5p expression was significantly lower in the 11q-deleted group compared to both the *MYCN* amplified and control groups. However, there was no significant difference in miR-708-5p expression between the *MYCN* amplified and control groups.

For miR-708-3p, expression was analyzed between two groups using an independent Welch's t-test, due to unequal sample sizes between groups (Welch, 1947). A t-test was preferred over a non-parametric alternative like a permutation test because with very small sample sizes, non-parametric tests can lack power and reliability (Fagerland, 2012). It showed a statistically significant difference between the control and IMR32. Expression of miR-708-3p in IMR-32 was compared to the control using Ct values, as recommended for more robust statistical handling of qPCR data (Yuan et al., 2006). The analysis showed a statistically significant difference, with IMR-32 exhibiting higher Ct values, indicating lower expression relative to the control. When interpreted through the  $\Delta\Delta\text{Ct}$  method, IMR-32 showed a negative  $\Delta\Delta\text{Ct}$ , suggesting upregulation compared to control. This apparent contradiction highlights how different analytical approaches can influence interpretation, especially when Ct differences are small.

However, the small sample sizes and assumptions of normality represent limitations that must be considered when interpreting the results, as statistical power is reduced and findings should be regarded as exploratory (Amrhein, Greenland, & McShane, 2019). Based on these significant differences and their association with key genetic features as *MYCN* amplification and 11q deletion, future studies with larger sample sizes could provide important clues for developing targeted therapies in neuroblastoma.

So further studies are needed to comprehensively understand how miRNA-708-5p and miR-7083p functions across different NB cell lines, which could provide valuable insights into its role as a potential therapeutic target or biomarker in NB.

## Conclusion

This study reveals distinct expression patterns of miR-708-5p and miR-708-3p across five NB cell lines. In all tested cell lines, miR-708-5p was expressed while miR-708-3p was detected only in the NB69, SH-SY-5Y, and IMR32 cell lines.

Non detectable miR-708-3p expression in SK-N-BE may result from epigenetic silencing, chromosomal alterations affecting the miRNA locus, or natural absence of expression under the studied conditions. However, in IMR-32 which also carries *MYCN* amplification, showed miR-708-3p expression and so further investigations are needed to specifically explore the regulatory relationship between *MYCN* amplification and miR-708-3p expression in NB, as direct regulation of miR-708-3p by *MYCN* has not been fully demonstrated.

In SK-N-AS cell line, which harbours an 11q deletion, miR-708-3p was not detected; probably the gene was lost due to 11q deletion. But miR-708-5p showed expression in this cell line, suggesting that partial or heterozygous 11q deletion left intact copies of the gene, allowing normal transcription. Also miR-708-5p is expressed in the cell line, the absence of detectable miR-708-3p (no Ct value) may be due to differences in the biogenesis, stability, or processing efficiency of the two mature miRNA strands derived from the same precursor. Typically, one strand (the guide strand, often the -5p) is preferentially incorporated into the RNA-induced silencing complex (RISC) and is more stable, while the other strand (the passenger strand, often the -3p) is degraded more rapidly and may be expressed at levels below the detection threshold. This strand selection and asymmetrical expression is well documented (Medley, Panzade, & Zinovyeva, 2021), indicating that the lack of miR-708-3p detection does not necessarily reflect gene deletion but rather natural differences in strand abundance. Genomic instability could also explain differences between cells. These show that genetic changes can affect miRNA expression in neuroblastoma and more studies are needed.

Statistical analysis of miRNA expression revealed significant differences among neuroblastoma cell lines. One-way ANOVA showed that miR-708-5p expression varied significantly between groups. Post hoc tests indicated that miR-708-5p expression was significantly lower in the 11q-deleted group compared to both the *MYCN* amplified and control groups. However, there was no significant difference in miR-708-5p expression between the *MYCN* amplified and control groups. Independent t-test was confirmed differences in miR-708-3p expression. Therefore, findings should be interpreted cautiously, considering the possibility of deviations from normality. Expanding future analyses with larger sample sets will be essential to confirm these results and to better understand their biological and clinical significance. Together, these findings suggest that miR-708-5p and miR-708-3p expression in neuroblastoma is shaped by underlying genetic changes and may provide important clues for future studies aimed at developing targeted therapies.

## **Ethical considerations**

Ethical considerations are essential in any scientific research, especially in biomedical studies where the materials used can have broad implications for both scientific integrity and societal impact. This study, which involves the use of commercially sourced NB cell lines, brings forth several important ethical dimensions. While it does not directly involve human participants or live animal subjects, the choice of using cell lines over primary human tissues or animal models reflects an ethical commitment to minimizing harm, upholding scientific rigor, and contributing to broader societal goals.

One of the key ethical advantages of using commercially available cell lines is the reduction of the need for live animal experimentation. The cell lines used in this project are procured from established and reputable sources, such as the ATCC, which ensures that these biological materials have been obtained ethically and with rigorous characterization. This approach aligns with the principles of the Three Rs—Replace, Reduce, and Refine—which advocate for the replacement of animal models whenever possible, the reduction of animals used in experiments, and the refinement of experimental procedures to minimize suffering (Russell & Burch, 1959). By using cell lines, this study effectively replaces the need for live animal models, thereby contributing to animal welfare (Kovalevich & Langford, 2016).

Furthermore, the use of cell lines enhances the reproducibility and rigor of scientific research. Commercially sourced cell lines are well-documented and widely validated, ensuring that experiments can be reliably replicated by other researchers. This reproducibility is a cornerstone of ethical research, as it promotes transparency, fosters scientific collaboration, and builds trust in the research process (Baker, 2016). By choosing established cell lines, this project contributes to the scientific community's ability to validate findings and advance the field of NB research.

From a societal perspective, this research holds the potential to benefit public health and advance medical knowledge. NB is a complex cancer, and studying its underlying mechanisms using cell-based models accelerates the pace of discovery in understanding disease progression and identifying potential therapeutic targets. The use of cell lines makes this research more cost-effective, allowing for a broader range of experiments and facilitating progress toward developing innovative therapies that could improve patient outcomes (Weiskirchen et al., 2023).

Additionally, there is a growing recognition of the environmental impact of biomedical research. Cell lines are less resource-intensive than live animal models, requiring fewer materials, energy, and infrastructure for maintenance. By reducing the overall environmental footprint of the research, this study contributes to a more sustainable scientific practice, an ethical consideration that is increasingly important in today's research landscape (Reardon, 2019).

Moreover, the integration of advanced tools such as artificial intelligence (AI) in analyzing large datasets generated from qPCR experiments presents another ethical dimension. AI enhances data accuracy and efficiency, helping to uncover complex patterns that would be difficult for humans to detect alone (Topol, 2019). This contributes to scientific honesty and precision in

reporting results, reducing the likelihood of errors or biases in the interpretation of data. To improve clarity, coherence, and the overall quality of presentation, advanced language and writing tools, including ChatGPT, were utilized. This approach ensures that the discussion is precise, accessible, and professionally communicated.

## Future perspectives

The findings of this study provide valuable insights into the expression dynamics of miR-708-5p and miR-708-3p across NB cell lines, but several aspects warrant further investigation. One key area for future research involves understanding the molecular mechanisms underlying the observed fluctuations in miRNA expression across different passages and cell lines. Significant differences in miRNA expression were observed between neuroblastoma cell lines, suggesting that genetic background and biological factors may influence miRNA regulation. Factors such as cell cycle stages, microenvironmental conditions, and genomic instability could contribute to these differences (Hwang & Mendell, 2006). Future studies could explore these factors in greater detail, potentially utilizing single-cell RNA sequencing or other high-throughput technologies to track expression at a higher resolution (Zhe Nian et al., 2024). This would help identify specific regulatory networks that modulate miRNA expression and their impact on cellular behavior in neuroblastoma.

Another important direction for future work is to investigate the functional role of miR-708-5p and miR-708-3p in NB. While the study primarily focused on expression patterns, understanding how these miRNAs contribute to processes like cell proliferation, apoptosis, and differentiation could reveal their potential as therapeutic targets (Condrat et al., 2020). Studies exploring the downstream genes regulated by miR-708 could further elucidate its role in NB pathogenesis and progression (Monteleone & Lutz, 2017). Additionally, it would be valuable to examine the potential of these miRNAs as biomarkers for disease prognosis or treatment response (Hwang & Mendell, 2006).

An important direction for future research is to include larger sample sets. Expanding the number of biological replicates and using this neuroblastoma cell lines would improve statistical power and make the findings more reliable. Finally, improving the stability and reproducibility of miRNA expression measurements across different cell lines and experimental conditions is crucial. Future studies should focus on refining methods for miRNA extraction and normalization, carefully considering passage-dependent variability and minimizing technical biases (Meyer et al., 2010). These improvements would strengthen the accuracy and consistency of miRNA expression data and ensure more reliable results for future neuroblastoma research.

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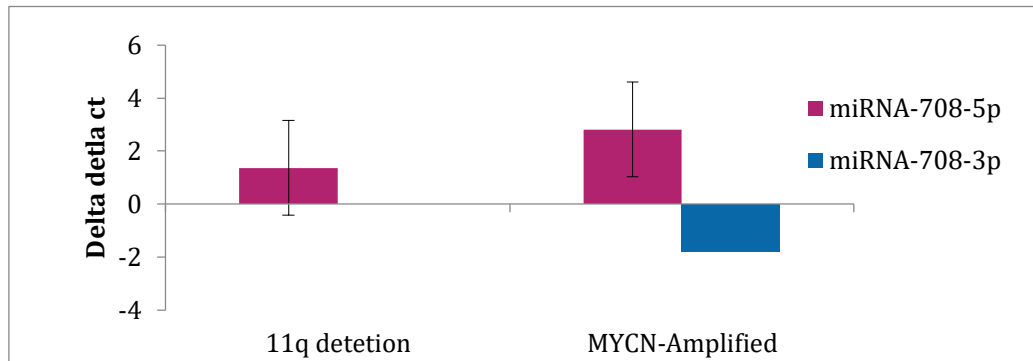
## Appendix I

Table1. Cell counts of cultured cell lines at 80-90% confluence counted by counting chamber

Cell lines	No of cell count		
	Passage replicate 1*	Passage replicate 2*	Passage replicate 3*
SH-SY-5Y	3.67*10 <sup>6</sup>	4.32*10 <sup>6</sup>	3.71*10 <sup>6</sup>
IMR32	5.97*10 <sup>6</sup>	4.32*10 <sup>6</sup>	5.45*10 <sup>6</sup>
NB69	3.30*10 <sup>6</sup>	3.89*10 <sup>6</sup>	4.35*10 <sup>6</sup>
SK-N-AS	3.50*10 <sup>6</sup>	3.53*10 <sup>6</sup>	3.24*10 <sup>6</sup>
SK-N-BE	2.30*10 <sup>6</sup>	1.34*10 <sup>6</sup>	2.10*10 <sup>6</sup>

Table2. Raw qPCR data for miR-708-5p and miR-708-3p across five neuroblastoma cell lines.

Cell Name	miR-708-5p			miR-708-3p			Endogenous Control		
	Ct1 1	Ct2	Ct3	Ct1	Ct2	Ct 3	Ct1	Ct2	Ct3
NB69 p11	25.82	25.7	25.93	36.79	36.65	37.09	30.19	30.23	30.0
NB69 p12	23.67	23.56	23.63	35.36	35.55	35.74	28.39	28.39	28.28
NB69 p13	24.52	24.35	24.42	35.49	35.57	35.66	30.72	30.59	30.74
SH-SY-5Y p11	26.38	26.13	26.18	36.7	36.72	36.69	28.1	28.16	28.07
SH-SY-5Y p12	25.69	25.72	25.78	34.32	34.45	34.57	28.7	28.7	28.72
SH-SY-5Y p13	25.98	26.35	26.43	36.66	36.84	36.47	27.72	27.46	27.78
IMR32 p6	25.67	25.69	25.66	36.08	35.86	36.23	30.45	30.61	30.49
IMR32 p7	26.31	26.37	26.21	34.43	34.13	34.28	29.34	29.41	29.35
IMR32 p8	26.44	26.41	26.33	No Ct	No Ct	No Ct	32.24	31.95	32.27
SK-N-AS p15	30.32	29.98	30.7	36.75	No Ct	No Ct	27.07	27.11	26.99
SK-N-AS p16	30.17	29.81	30.7	No Ct	No Ct	No Ct	27.92	27.86	27.82
SK-N-AS p17	30.52	30.05	30.13	No Ct	No Ct	No Ct	26.84	26.87	26.86
SK-N-BE p6	26.38	26.14	26.62	36.51	No Ct	No Ct	29.42	29.44	29.16
SK-N-BE p7	26.95	26.98	26.83	No Ct	No Ct	No Ct	27.77	27.85	27.86
SK-N-BE p8	26.95	26.94	26.95	No Ct	No Ct	No Ct	29.91	29.89	29.55



**Figure 1.** Relative expression ( $\Delta\Delta Ct$ ) of miR-708-5p and miR-708-3p in neuroblastoma cell lines grouped by *MYCN* amplification and 11q deletion status. Expression levels were normalized to the reference gene RNU6B, and SH-SY-5Y and NB69 were used as calibrator controls ( $\Delta\Delta Ct = 0$ ).

miR-708-5p showed lower expression in both *MYCN*-amplified ( $\Delta\Delta Ct = 0.20 \pm 0.73$ ) and 11q-deleted ( $\Delta\Delta Ct = 6.59 \pm 0.32$ ) groups. miR-708-3p expression was detectable only in control and *MYCN*-amplified lines, with higher expression observed in IMR-32 ( $\Delta\Delta Ct = -1.78 \pm 0.32$ ); it was undetectable in 11q-deleted lines.